SHORT COMMUNICATION

Red-fleshed apple as a source for functional beverages

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Rupasinghe, H. P. V., Huber, G. M., Embree, C. and Forsline, P. L. 2010. **Red-fleshed apple as a source for functional beverages**. Can. J. Plant Sci. **90**: 95–100. The potential of anthocyanin pigments in fruits and vegetables to provide protection against oxidative damage is well known. Cyanidin-3-*O*-galactoside is a naturally occurring red pigment commonly found in skin of apples but also present in flesh of certain crab apple and apple genotypes. The purpose of this study was to investigate the cyanidin-3-*O*-galactoside concentration and antioxidant capacity of juice made from 14 genotypes of red-fleshed apples in comparison to three white-fleshed apple cultivars and three commercial apple juices. Cyanidin-3-*O*-galactoside was found only in the juice made from red-fleshed apple genotypes with the highest concentrations (39 mg L^{-1}) in a crab apple Roberts Crab. The antioxidant capacity measured using the Folin-Ciocalteu, the ferric reducing antioxidant power (FRAP), and the oxygen radical absorbance capacity (ORAC) assays were the greatest in juice prepared from the red-fleshed genotypes Babine and *Malus pumila* Niedzwetzkyana, a red-fleshed crab apple genotype. The antioxidant capacity measures were strongly correlated with each other; however, there was no correlation between the concentration of cyanidin-3-*O*-galactoside and the antioxidant capacity measures. The juice quality parameters °Brix and titratable acidity values were not significantly different among the juices made from the red-fleshed apples, commercial apples and commercial apple juice products.

Key words: Red-fleshed apple, *Malus* species, antioxidants, cyanidin-3-*O*-galactoside, Folin-Ciocalteu, FRAP, ORAC, functional beverage

Rupasinghe, H. P. V., Huber, G. M., Embree, C. et Forsline, P. L. 2010. **Fabrication de boissons fonctionnelles à partir des pommes à chair rouge**. Can. J. Plant Sci. **90**: 95–100. On sait que les pigments de l'anthocyanine présents dans les fruits et les légumes peuvent prémunir l'organisme contre les dommages dus à l'oxydation. Le cyanidine-3-*O*-galactoside est un pigment rouge qu'on trouve couramment à l'état naturel dans la pelure des pommes, mais il existe aussi chez divers génotypes de pommetier décoratif et de pommier. L'étude devait établir la concentration de ce pigment et le pouvoir antioxydant du jus de 14 génotypes de pomme à chair rouge, comparativement au jus de trois cultivars à chair blanche et à trois jus de pomme commerciaux. Le cyanidine-3-*O*-galactoside n'a été dépisté que dans le jus des pommes à chair rouge, la plus forte concentration (39 mg L^{-1}) ayant été relevée chez le pommetier Roberts Crab. Le pouvoir antioxydant mesuré selon l'indice de Folin-Ciocalteu, le pouvoir de réduction antioxydant de l'ion ferrique (FRAP) et la capacité d'absorption des radicaux oxygénés (ORAC) sont plus élevés dans le jus des pommes à chair rouge Babine et des pommettes à chair rouge *Malus pumila* Niedzwetzkyana. Les mesures du pouvoir antioxydant sont étroitement corrélées entre elles; néanmoins, il n'existe aucune corrélation entre la concentration de cyanidine-3-*O*-galactoside et le dosage du pouvoir antioxydant. Les paramètres de qualité du jus (nombre de degrés Brix et acidité totale) ne varient pas significativement entre le jus des pommes à chair rouge, le jus des cultivars commerciaux ou le jus de pomme vendu dans le commerce.

Mots clés: Pomme à chair rouge, espèce du genre *Malus*, antioxydants, cyanidine-3-*O*-galactoside, Folin-Ciocalteu, FRAP, ORAC, boisson fonctionnelle

The lower incidence of cancer and cardiovascular diseases associated with diets high in fruit is potentially due to the presence of phytochemicals such as polyphenolics (Steinmetz and Potter 1996). Apples are common in the North American diet and represent the second highest source of dietary polyphenolics among fruits, next to oranges (Chun et al. 2005). The health benefits of the polyphenolic compounds found in apples are well documented (Boyer and Liu 2004), and apple juice has been shown to possess anti-ulcerative (Hamauzu et al. 2008) and anticancer (Barth et al. 2007) properties among many others.

Abbreviations: **FRAP**, ferric reducing antioxidant power; **ORAC**, oxygen radical absorbance capacity

Anthocyanins are found in the flesh of some apple genotypes, such as crab apples, but are not commonly found in the flesh of commercial apple cultivars (Khanizadeh et al. 2008). Anthocyanins have been demonstrated to posses a wide range of potential health benefits to humans and animals, such as reducing the risk of cardiovascular diseases (Andriambeloson et al. 1998), diabetes (Cohen-Boulakia et al. 2000), allergies (Borissova et al. 1994), and benefits to visual acuity in dark conditions (Nakaishi et al. 2000). The most abundant anthocyanin found in apple skin is cyanidin-3-*O*-galactoside (van der Sluis et al. 2004).

The purpose of this research was to examine the potential use of red-fleshed apples for developing a unique red-colored functional beverage with high antioxidant capacity. The concentration of cyanidin-3-*O*-galactoside, antioxidant capacity, and selected quality attributes of juices made from red-fleshed apples were compared with those of juices made from commercial cultivars and commercial juice products.

Juice Preparation

Red fleshed apples were collected from the Plant Genetic Resources Unit of the USDA Agricultural Research Services, Geneva, NY (PI series; http://www.ars-grin.gov/npgs/searchgrin.html) and the Atlantic Food and Horticulture Research Centre of Agriculture and Agri-Food Canada, Kentville, NS, during the 2007 harvest season. Apples of three commercial cultivars and juice products (unsweetened) were purchased from local market. The randomly grouped apples from each genotype were rinsed in deionized water and then chopped into 1–2 cm^3 pieces. The apple pieces were pressed using a manual fruit press (Musca Fruit Pressing and Supplies Limited, Ottawa, ON). The juice samples were centrifuged at 5000 rpm for 10 min (model 300, Precision Durafuge, Colonial Scientific, Richmond, VA) before use for the chemical analyses.

High Performance Liquid Chromatography and Mass Spectrometry Analysis

The HPLC system consisted of a Waters Alliance 2695 Separation Module that consisted of a quaternary pump and autosampler. The reverse phase column used was a Phenomenex Luna C_{18} (150 mm × 2.1 mm, 5 μm) with a Waters X-Terra MS C_{18} guard column. A previously reported method (Rupasinghe et al. 2008) was used for the analysis of cyanidin-3-*O*-galactoside. Briefly, a linear gradient elution was carried out with 5% formic acid in water (Solvent A) and 5% formic acid in methanol (Solvent B) at a flow rate of 0.35 mL min^{-1} as follows: time *t* (min); (*t*, A%): (0, 90%), (10, 70%), (17, 60%), (21, 48.8%), (26, 36%), (30, 10%), (31, 90%), (37, 90%). MS/MS analyses were preformed on a Waters Micromass Quattro *micro* API triple quadrupole mass spectrometer. Electrospray ionization in positive ion mode (ES+; capillary voltage 3500 V) was used with a flow rate of 600 L h^{-1} maintained for the nebulizer gas (N_2, 375°C). Cyanidin-3-*O*-galactoside was identified and quantified using multiple reaction monitoring mode (m/z 449 → 287) in comparison with a standard. In the multiple reaction monitoring experiment, both quadrupoles were operated at unit resolution. The limit of detection of cyanidin-3-*O*-galactoside was 0.05 mg L^{-1}.

Determination of Total Phenol Content – Folin-Ciocalteu Assay

The Folin-Ciocalteu assay, an assay developed by Singleton and Rossi (1965), was used to estimate the total phenols present in the apple juice. The assay was modified for use with a 96-well, 200 μL high throughput microplate reader, the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC). Briefly, 20 μL of the apple extract was mixed with 100 μL of 0.2 N Folin-Ciocalteu reagent in the microplate wells of the clear 96-well microplates (COSTAR 9017, Fisher Scientific, Ottawa, ON) and left to stand. After 5 min, 80 μL of a 7.5% sodium carbonate solution was added and the microplate was covered for 2 h at ambient temperature before reading at 760 nm. The total phenol measure was calculated using gallic acid standards made to 1.18, 2.35, 3.53, 4.70, 5.88, and 8.82 μM concentrations. The solutions were made fresh under reduced light conditions and the reaction was carried out under dark conditions.

Ferric Reducing Antioxidant Power Assay

The ferric reducing antioxidant power (FRAP) assay was also used to determine the electron donating potential of the juice based on the assay described by Benzie and Strain (1996). The FRAP assay was also modified for a 96-well microplate reader with an injection port system (BMG Labtech, Durham, NC). The working reagent, consisting of 300 mM acetate buffer (pH 3.6), 1 mM 2,4,6-Tris(2-pyridyl)-s-triazine solution, and 20 mM ferric chloride, was combined in the ratio 10:1:1 directly before analysis and preheated to 37°C. Twenty microlitres of each sample or standard was placed in the wells of the 96-well clear polystyrene microplate (COSTAR 9017, Fisher Scientific, Ottawa, ON) and 180 μL of the working reagent was injected by the port of the FLUOstar OPTIMA plate reader. The absorbance was read at 593 nm after a 6 min reaction time and antioxidant capacity was calculated based on Trolox standards at the concentrations, 5, 10, 25, 75, 150, and 300 μM.

Oxygen Radical Absorbance Capacity Assay

The hydrophilic oxygen radical absorbance capacity (ORAC) assay was performed based on Huang et al. (2002). The fluorescein sodium salt (0.957 μM), as well as samples and standards, was diluted in 75 mM phosphate buffer (K_2HPO_4/ NaH_2PO_4, pH 7). Thirty-five microlitres of the sample or Trolox standard and 130 μL of the fluorescein probe were combined in the wells of the black 96-well polystyrene microplate

(COSTAR 3915, Fisher Scientific, Ottawa, ON) and the plate was warmed to 37°C for 5 min. The injection port was used to inject 35 μL of 150 mM pre-warmed (37°C) AAPH into the wells. The plate was maintained at 37°C for the duration of the analyses (approximately 45 min) with excitation and emission readings every 45 s for the first 2 min then at every 2 min for the remaining 43 min. Excitation of the reaction mixture was at 490 nm and the emission was read at 510 nm. The antioxidant capacity of the samples was calculated as Trolox equivalents using a quadratic relation developed from area under the fluorescence decay curves for standards made to 5, 10, 25, 50, 75 μM concentrations.

Juice Quality Measurements

The sugar content of the juices, as total soluble solids, was measured using a hand-held digital refractometer (Model 300016, Sper Scientific Ltd, Scottsdale, AZ), the pH was measured using a pH meter, and the titratable acidity was measured using a titrator (Model 785 DMP Titrino, Metrohm, Oberdorfstr, CH-9100 Herisau, Switzerland).

Experimental Design and Analysis

For the juice prepared in the laboratory, replicates consisting of 6 to 12 apples were pressed separately from randomly grouped apples. Three separate product units of each of the commercial juices formed the replicates. Analyses of variance and Tukey's multiple means comparisons were performed using SAS software V8 (SAS Institute, Inc., Cary, NC). Pearson correlation analyses were performed using MINITAB 14.1 (State College, PA).

Cyanidin-3-*O*-galactoside Concentration and Antioxidant Capacity

Cyanidin-3-*O*-galactoside, the most predominant anthocyanin of the red-skinned apples (Seeram et al. 2003), was detected in the juice prepared from all red-fleshed apples. The juice prepared from the commercial cultivars and the commercial juice products did not contain cyanidin-3-*O*-galactoside (Table 1). Similar to the present findings, trace concentrations or the absence of cyanidin-3-*O*-galactoside in apple juice and cider are reported (van der Sluis et al. 2004; Del Campo et al. 2006). Red-fleshed crab apple Roberts Crab (PI 437057)

Table 1. Antioxidant capacity and cyanidin-3-*O*-galactoside concentration for juices of the red-fleshed and white-fleshed apple genotypes and commercial apple juice products

Source of apple juice	Genotype or product name	Folin-Ciocalteu (mg GAE[z] L^{-1})	FRAP (g TE[z] L^{-1})	ORAC (g TE L^{-1})	Cyanidin-3-*O*-galactoside (mg L^{-1})[y]
Red-fleshed apple	*Malus pumila* Niedzwetzkyana	21.9*a*	3.12*a*	10.4*b*	12.5
	Malus marjorensis "Formosa"	8.72*cdef*	0.857*bcd*	3.04*de*	1.25
	Roberts Crab (PI 437057)	7.74*cdef*	0.812*cd*	4.08*cde*	38.9
	Otterson (PI 590178)	12.2*bc*	1.41*b*	5.10*cd*	8.2
	Red Sauce (PI 589087)	5.02*ef*	0.490*d*	1.97*e*	12.9
	Cranberry (PI 589180)	6.28*efd*	0.497*d*	2.27*de*	3.15
	Babine (PI 148490)	20.6*a*	3.14*a*	15.0*a*	2.5
	Okanagan (PI 148708)	6.82*cdef*	0.682*cd*	5.38*cd*	0.2
	M. Marjorensis "Formosa" (PI 589411)	6.30*efd*	0.603*cd*	4.33*cde*	3.25
	Form 35(33-01) E7-NYAES (PI 613967)	4.66*ef*	0.424*d*	2.52*de*	0.25
	Redford (PI 589056)	10.9*bcd*	1.1*bc*	6.45*c*	16.5
	Rose Bud (PI 589130)	5.61*efd*	0.510*d*	2.64*de*	0.45
	Manito (PI 589155)	4.26*ef*	0.344*d*	2.46*de*	0.2
	KAS-9	3.81*f*	0.385*d*	1.92*e*	0.35
Commercial apple	Cortland	5.65*efd*	0.406*d*	2.56*de*	ND[x]
	McIntosh	5.99*efd*	0.491*d*	2.66*de*	ND
	Delicious	5.69*efd*	0.485*d*	2.68*de*	ND
Commercial apple Juice products	Juice cartoon	8.30*cdef*	0.444*d*	1.11*e*	ND
	Frozen juice product	9.75*bcde*	0.665*cd*	1.12*e*	ND
	Canned Juice product	14.4*b*	0.858*bcd*	2.42*de*	ND
Statistics	Mean	8.73	0.886	4.01	5.02
	Median	6.99	0.603	2.66	0.400
	Among genotypes	$P<0.0001$	$P<0.0001$	$P<.0001$	N/A
	Among categories	$P=0.489$	$P=0.501$	$P=0.241$	N/A

[z]GAE, gallic acid equivalents; TE, Trolox equivalents.
[y]Mean and median of concentration of cyanidin-3-*O*-galactoside is based on red-fleshed apples only.
[x]ND, not detected.
a–f Means followed by different letters within the same column represent significant differences according to Tukey's multiple mean comparisons ($P<0.05$).

and *Malus pumila* Niedzwetzkyana (a crab apple genotype) as well as the red-fleshed cultivars Redford (PI 589056) and Red Sauce (PI 589087) had significantly higher concentrations of cyanidin-3-*O*-galactoside than others. However, the concentration of cyanidin-3-*O*-galactoside varied greatly among red-fleshed apple genotypes. Research has shown that anthocyanin concentration in apples is highly cultivar dependent, as well as heavily influenced by season and environment (Lata et al. 2005). For example, the expression of a gene encoding R2R3 MYB transcription factor, which regulates anthocyanin biosynthesis in apple skin is induced by exposure to light (Takos et al. 2006).

Interestingly, juice prepared from two of the red-fleshed apple genotypes, *Malus pumila* Niedzwetzkyana and the cultivar Babine (PI 148490), exhibited significantly higher antioxidant capacity, determined by three antioxidant capacity assays, Folin-Ciocalteu, FRAP, and ORAC (Table 1). In comparison with the juice prepared from the commercial apple cultivars and the commercial juice products, juice of *Malus pumila* Niedzwetzkyana and Babine had over fivefold greater ORAC and FRAP values. However, antioxidant capacity of red-fleshed apples varied among red-fleshed genotypes compared with that of commercial cultivars. The juices of commercial cultivars were below the median antioxidant capacity of the Folin-Ciocalteu and FRAP assays, whereas, the commercial juice products were, for the most part, above the median antioxidant capacity. For the ORAC assay, the commercial juice product ranked lower and the juices of commercial cultivar ranked higher than the median antioxidant capacity.

Juice Quality Measurements

Juice quality was determined by total soluble solids, titratable acidity, and pH in order to compare the acceptability of a red-fleshed juice with products in the market, as well as three selected commercial cultivars. The TSS, pH, and TA values ranged between 9.4 and 17.5, 3.05 and 4.06 and 0.242 and 1.28, respectively. However, there was no difference in juice characteristics among the three juice categories: red-fleshed, commercial cultivars, and commercial juice products, although there were significant differences among the genotypes/juice product type (Table 2).

Correlation Analyses

The juice from red-fleshed cultivars with the highest concentrations of cyanidin-3-*O*-galactoside, Roberts Crab, Redford, and Red Sauce, did not rank the highest among the juices for antioxidant capacity. In fact, the cyanidin-3-*O*-galactoside concentrations were not strongly correlated with any of the antioxidant capacity measures, Folin-Ciocalteu, FRAP, or ORAC, according to Pearson correlation analyses. However, the antioxidant capacity measures were highly correlated with one

Table 2. General measures of juice quality for red-fleshed apple, white-fleshed apple, and commercially produced apple juice

Source of apple juice	Genotype or product name	Total soluble solids (%)	pH	Titratable acidity (% malic acid)
Red-fleshed apple	*Malus pumila* Niedzwetzkyana	12.0*bcd*	3.05*j*	1.28*a*
	Malus marjorensis "Formosa"	12.5*abcd*	3.23*fghi*	0.626*cde*
	Roberts Crab (PI 437057)	12.2*bcd*	3.73*b*	0.598*cde*
	Otterson (PI 590178)	11.3*bcd*	3.99*a*	0.395*efg*
	Red Sauce (PI 589087)	15.5*abc*	3.06*ij*	1.02*b*
	Cranberry (PI 589180)	17.5*a*	3.64*bc*	0.792*bc*
	Babine (PI 148490)	16.4*ab*	3.19*ghij*	0.719*cd*
	Okanagan (PI 148708)	10.5*cd*	3.51*cd*	0.390*efg*
	M. Marjorensis "Formosa" (PI 589411)	12.2*bcd*	3.16*hij*	0.783*bc*
	Form 35(33-01) E7-NYAES (PI 613967)	9.40*d*	3.41*def*	0.307*g*
	Redford (PI 589056)	13.7*abcd*	3.24*fgh*	0.719*cd*
	Rose Bud (PI 589130)	12.9*abcd*	3.30*efgh*	0.469*defg*
	Manito (PI 589155)	13.0*abcd*	3.38*def*	0.618*cde*
	KAS-9	10.9*cd*	3.50*cd*	0.328*fg*
Commercial apple	Cortland	12.8*abcd*	3.64*bc*	0.374*efg*
	McIntosh	14.5*abcd*	4.06*a*	0.242*g*
	Delicious	10.9*cd*	3.47*cde*	0.488*defg*
Commercial apple Juice products	Juice cartoon	10.8*cd*	3.64*bc*	0.399*efg*
	Frozen juice product	11.3*bcd*	3.61*bc*	0.414*efg*
	Canned Juice product	10.8*cd*	3.34*defg*	0.575*cdef*
Statistics	Mean	12.5	3.45	0.577
	Median	12.0	3.42	0.544
	Among genotypes	$P=0.0003$	$P<0.0001$	$P<0.0001$
	Among categories	$P=0.374$	$P=0.143$	$P=0.170$

a–j Means followed by different letters within the same column represent significant differences according to Tukey's multiple mean comparisons ($P<0.05$).

another: Folin-Ciocalteu and FRAP, $r=0.94$ ($P=0.001$); Folin-Ciocalteu and ORAC, $r=0.76$ ($P=0.001$); and FRAP ($P=0.001$) and ORAC, $r=0.89$ ($P=0.001$). Roberts Crab had high concentration of cyanidin-3-*O*-galactoside, whereas the cultivar Babine and *Malus pumila* Niedzwetzkyana had the highest antioxidant capacity according to all three antioxidant capacity assays. Babine showed fairly low levels of cyanidin-3-*O*-galactoside, although more than the commercial cultivar and commercial juice products. The lack of strong correlation between concentration of cyanidin-3-*O*-galactoside and antioxidant capacity could be due to the presence of other antioxidant compounds in juice. Seventeen phenolic compounds that belong to flavan-3-ols, hydroxycinnamates, flavonols, and dihydrochalcones were identified in 23 English apple ciders (Marks et al. 2007). Also, red color of juice of red-fleshed apples may also be due to anthocyanins other than cyanidin-3-*O*-galactoside. Recently, another anthocyanin, cyanidin-3-*O*-glucosyl rutinoside, has been identified in methanol extracts of red-fleshed crab apples using nuclear magnetic resonance (Mulabagal et al. 2007). Titratable acidity was correlated with the antioxidant capacity measures, Folin-Ciocalteu ($r=0.51$, $P=0.001$), FRAP assay ($r=0.55$, $P=0.001$), and ORAC assay ($r=0.43$, $P=0.005$). This is likely due to the presence of organic acids such as malic acid, chlorogenic acid and its derivatives, which may contribute to the antioxidant capacity (Marks et al. 2007).

In conclusion, the results revealed that juice prepared from specific red-fleshed apple genotypes had higher antioxidant capacity than that of the commercial cultivars, as well as commercial juice products. The antioxidant capacity was exceptionally high for the juice made from the *Malus pumila* Niedzwetzkyana and the cultivar Babine and the concentration of cyanidin-3-*O*-galactoside was greatest in the red-fleshed genotypes Roberts Crab, Redford, Red Sauce and *Malus pumila* Niedzwetzkyana. Considering the acceptable juice quality parameters, attractive color, and greater antioxidant capacity, these underutilized red-fleshed apple genotypes warrant further investigation as sources of a functional fruit beverage to improve human health.

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